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(57) Abstract

The present invention provides a polypeptide having or including an amino acid sequence substantially corresponding t all or a portion of the amino acid sequence set out in Figure 1 (SEQ ID NO: 1) and derivatives and fragments thereof having bac teriocin and/or bacteriocin immunity activity.

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BACTERIOCIN

The present invention relates to a novel bacteriocin, its isolation, synthesis and use.

Bacteriocins are peptides or proteins released by bacteria which show bactericidal activity towards both the producing strain and/or other bacteria.

The producing organism will generally carry a gene coding for an immunity factor which provides resistance to the bacteriocin, and the producing organisms are usually only affected by their own bacteriocin at high cellular concentrations.

Due to their potential use as antibacterial agents, bacteriocins have been the subject of intensive research. In recent years there has in particular been considerable interest in bacteriocins isolated from lactic acid bacteria (LAB), in view of their potential utility in the food and brewing industries, in particular the dairy industry.

Thus for example, their selective bactericidal activity renders LAB bacteriocins suitable for use in cheese or yoghurt manufacture or in beer or distillery fermentations.

The LAB bacteriocins appear to be structurally quite different from other bacteriocins eg. the colicins of Eschericia coli. LAB bacteriocins are usually small peptides, seldom containing more than 60 amino acids, while colicins are proteins of 300-800 amino acids. Based on their structure, LAB bacteriocins may be divided into two groups. The first group contains the so-called lantibiotics, which have been known for a long time and include in particular the known bacteriocin nisin, (see for example Gross, et al J. Am. Chem. Soc. 93: 4634-4635, 1971 and Hurst, Adv. Appl. Microbiol. 27: 85-123, 1981). Lantibiotics consist of a polypeptide chain in which certain amino acids have been post

translationally modified. The modified amino acids include lanthionine and methyllanthionine, and their precursors dehydroalanine and dehydrobutyrine. Among the antibiotics, nisin is by far the most studied although three new LAB antibiotics have recently been purified and characterised (Mørtvedt et al., J. Gen. Microbiol. 136: 1601-1607, 1990 and Appl. Environ. Microbiol 37: 1829-1834, 1991; Piard et al., Appl. Environ. Microbiol. 58: 279-284, 1992).

The second group of LAB bacteriocins contains those that consist of one short unmodified polypeptide chain, such as lactococcin A (Holo et al., J. Bacteriol, 173: 3879-3887, 1991; Van Belkum et al., Appl. Environ. Microbiol. 57: 492-498, 1991), leucocin A-UAL 187 (Hastings et al., J. Bacteriol. 173: 7491-7500, 1991), lactacin F (Murlane et al., J. Bacteriol. 173: 1779-1788, 1991 and Appl. Environ. Microbiol 57: 114-121, 1991), pediocin PA-1, sakecin P, and curvacin A. These bacteriocins contain between 35 and 60 amino acid residues, and have a high isoelectric point, often above 10. Many of these bacteriocins share significant amino acid sequence homology over relatively large regions, suggesting that these regions may be of importance for activity. Lactococcin A, which does not share any apparent amino acid sequence homology with other isolated LAB bacteriocins, has been shown to induce cell death by permeabilizing the membrane of susceptible cells (Van Belkum, et al., J. Bacteriol. 173: 7934-7941, 1992).

The antimicrobial activity of the bacteriocins that have so far been studied is due to the action of a single peptide. We have now found, however, a novel lactococcal bacteriocin, which we have termed lactococcin G, whose activity depends on the complementary action of two distinct peptides.

The novel bacteriocin of the invention has been isolated from Lactococcus lactis and purified and

bacteriocin activity was found to be associated with two peptides, designated α and β, the α peptide of which may appear in two forms, α_1 and α_2, which are identical and may differ only in conformation. A corresponding immunity factor has also been identified.

In one aspect the present invention thus provides a polypeptide having or including the amino acid sequence substantially corresponding to all or a portion of the amino acid sequence set out in Figure 1 (SEQ ID NO: 1) and derivatives and fragments thereof having bacteriocin and/or bacteriocin immunity activity.

Preferably, the said amino acid sequence substantially corresponds to the sub-sequences identified as lag A, lag B and lag C in Figure 1. Peptides α and β are believed to be expressed in a "pro" form which is processed to the mature α or β peptide.

According to a further aspect the present invention also provides a polypeptide having or including the amino acid sequence

α_1 and α_2 (SEQ ID NO: 2):

N Gly Thr Trp Asp Asp Ile Gly Gln Gly
Ile Gly Arg Val Ala Tyr Trp Val Gly
Lys Ala Met Gly Asn Met Ser Asp Val
Asn Gln Ala Ser Arg Ile Asn Arg Lys
Lys Lys His C

and/or

β (SEQ ID NO: 3):

N Lys Lys Trp Gly Trp Leu Ala Trp Val
Asp Pro Ala Tyr Glu Phe Ile Lys Gly
Phe Gly Lys Gly Ala Ile Lys Glu Gly
Asn Lys Asp Lys Trp Lys Asn Ile C

and derivatives and fragments thereof having bacteriocin activity.

In a still further aspect, the invention also provides a polypeptide having or including the amino acid sequence

Leu	Phe	Asn	Asn	Ile	Val	Val	Phe	Ile
Asn	Phe	Leu	Ser	Phe	Val	Phe	Ile	Leu
Val	Gly	Val	Asp	Ile	Lys	Tyr	Asn	Asp
Asn	Arg	Ile	Lys	Ile	Val	His	Val	Thr
Phe	Phe	Ile	Ser	Phe	Ile	Leu	Val	Met
Leu	Thr	Ser	Leu	Ile	Ser	His	Asn	Ser
Ile	Ala	Tyr	Ser	Leu	Ser	Gln	Ile	Leu
Glu	Ile	Leu	Cys	Ile	Ile	Cys	Ile	Leu
Leu	Leu	Phe	Tyr	Ile	Leu	Lys	Lys	Thr
Asn	Ser	Leu	Ser	Asn	Arg	Ala	Asn	Val
Val	Phe	Ile	Ile	Phe	Ile	Val	Thr	Gln
Val	Ile	Ile	Ile	Ile	Asn	Gln	Leu	Phe
Ile	Arg							

(SEQ ID NO: 4) and derivatives and fragments thereof having bacteriocin immunity factor activity.

Viewed from another aspect, the invention also provide a bacteriocin comprising peptide chains α and β as designated above or combinations of active fragments or derivatives thereof and having or including the amino acid sequences set out above. In such combinations, peptide α as designated herein may be either peptide α_1 or α_2 or a mixture of the two.

The terms "bacteriocin activity" and "active" are used to denote activity in inhibiting the growth of bacterial species, for example a lactococcus test organism. Bacteriocins may kill or inhibit the growth of bacteria by a number of mechanisms including lysis and it is not intended to limit to any particular type of bactericidal activity. Active polypeptides and derivatives or fragments include those which, whilst on their own do not exhibit bacteriocin activity, contribute to such activity when combined with the

complementary (α or β) peptide or its fragment or derivative.

The term "bacteriocin immunity factor activity" denotes activity in providing immunity to the bacteriocin of the invention.

Derivative sequences included within the scope of the invention include functionally-equivalent sequences modified by single or multiple amino acid substitution, addition and/or deletion and also sequences where the amino acids have been chemically modified, including by glycosylation or deglycosylation. By "functionally equivalent" is meant amino acid sequences having essentially equivalent bacteriocin activity. Such functionally equivalent derivatives may occur as natural biological variations or may be prepared using known techniques, for example functionally equivalent recombinant polypeptides may be prepared using the known techniques of site-directed mutagenesis, random mutagenesis, or enzymatic cleavage and/or ligation of amino acids.

As mentioned above, modification of the amino acid sequences to obtain functionally-equivalent derivative sequences may be amino acid substitution, as long as the activity of the polypeptide is not affected. Thus for example, an amino acid may be replaced by another which preserves the physicochemical character of the polypeptide eg. in terms of charge density, hydrophilicity/hydrophobicity, size and configuration. For example A may be replace by G or vice versa, V by A, L or G; K by R; S by T or vice versa; E by D or vice versa; and Q by N or vice versa.

Generally, the substituting amino acid has similar properties eg. hydrophobicity, hydrophilicity, electronegativity, bulky side chains etc. to the amino acid being replaced.

"Addition" derivatives include amino and/or carboxyl terminal fusions, for example by addition of

amino acid sequences of up to 300 eg. up to 200 or 100 residues, as well as intrasequence insertions of single or multiple amino acids.

Insertional amino acid sequence derivatives are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Preferably, deletions or insertions are made in adjacent pairs eg. a deletion of two residues or insertion of two molecules. In all cases the proviso is that the modification preserves the activity of the polypeptide.

Derivative sequences falling within the scope of the invention may thus include for example amino acid sequences having at least 60%, eg. at least 70% or 80% sequence homology with the sequences of peptides α_1, α_2 or β set out above. It should be noted however that functionally-equivalent derivative peptides may exhibit overall sequence homology below the given figures, but may still fall within the scope of the present invention where they have conserved regions of homology.

Bacteriocin activity, as mentioned above, requires the complementary action of the α and β peptides, α_1 being more effective when combined with β, than α_2 and the novel bacteriocin as provided according to the invention preferably comprises both α and β peptides.

In tests on Lactococcus Lactis subsp. Lactis 1403 indicator cells, the concentrations of peptides α_1 and β which inhibited cell growth by 50% were found to be 0.15 and 0.02 nM when the complementing peptide was present in excess. When neither was in excess the concentrations were respectively 0.3 and 0.04 nM. It thus appears that roughly 8 times more of α_1 peptide is needed, and whilst not wishing to be bound by theory, it is possible that α and β peptides interact in an

approximately 8 to 1 ratio in effecting bacteriocin activity.

A further aspect of the invention thus includes bacteriocin comprising peptides α and β or fragments or derivatives thereof in a ratio of 5-10 to 1, preferably 7-9 to 1, especially 8 to 1 respectively.

As judged by its amino acid sequence, peptide α_1 has an isoelectric point of 10.9, extinction coefficient of 1.3×10^{-4} M^{-1} cm^{-1}, and a molecular weight of 4,346 (39 amino acid residues long). Similarly, peptide β has an isoelectric point of 10.4, extinction coefficient of 2.4×10^{4} M^{-1}, and a molecular weight of 4110 (35 amino acid residues long). Molecular weights of 4,376 and 4,109 for α_1 and β, respectively, were obtained by mass spectrometry. The N-terminal half of both the α and β peptides may form amphiphilic α-helices, suggesting that the peptides are pore-forming toxins that create cell membrane channels through a "barrel-stave" mechanism and the novel bacteriocin of the invention may exert its bactericidal effect in this manner. The C-terminal half of both peptides consists largely of polar amino acids.

Peptides α_1 and α_2 are believed to represent different forms of the same peptide. Amino acid sequence determination suggests that the amino acid sequences for peptides α_1 and α_2 are identical and the two peptides were separated by their different behaviour during purification, particularly in reverse phase chromatography. In particular upon rechromatography of purified α_1 on a reverse phase column, a certain proportion eluted as α_2, suggesting that peptide α_2 derives from α_1. Peptides α_1 and α_2 thus appear to represent the same gene product, but may differ in their configuration in a manner which results in α_2 having a slightly lower affinity for the reverse phase column, and reduced bacteriocin activity when combined with β, than peptide α_1.

A further aspect of the invention provides a

composition comprising bacteriocin according to the present invention, together with at least one of a carrier, and/or diluent, or excipient.

Such compositions may have a number of uses, particularly in microbiological processes and the carrier, diluent or excipient may be any such conventional material, chosen according to the proposed end use, for example a sterile liquid medium, buffer etc.

The novel bacteriocin of the invention may be used in industrial processes in which microbial species eg. lactobacteria such as lactococcus, are employed, for example in cheese and yoghurt manufacture.

The bacteriocin of the invention may be employed in fully or partially purified form or directly on the culture supernatant. The latter may be advantageous in certain circumstances, for example when the bacteriocin is to be used to selectively kill clostridia, as described below.

It may in certain cases be desirable to kill or arrest the growth of lactobacterial species for example, lactococcus in cheese ripening, and the new bacteriocin may thus be of particular application in the production of cheese, or other diary products such as yoghurt.

Alternatively, the bacteriocin may be used to kill undesired or contaminating bacterial cells in various preparations. Such cells may be lactobacteria or they may be other bacterial strains, for example species of Bacillus (eg. B. Cereus) or Clostridium eg. C. tyrobutyricum.

Thus, for example since certain bacteria, for example some Gram-negative bacteria, may be resistant to bacteriocins, negative selection is possible by using the bacteriocin according to the invention to remove certain cells, for example clostridia or strains of L.lactis or other lactobacteria, from mixed cell populations e.g. in starter cultures for fermentation.

Where the productive strain of L.lactis is used as the sole or principle organism in an industrial process such as cheese or yoghurt production, addition of the bacteriocin of the invention to the starter culture serves to eliminate foreign organisms and may be effective against, for example, spore forming clostridia or unwanted strains of L.lactis, or other lactobacteria.

The bacteriocin may advantageously be added to a cheese or yoghurt fermentation at a relatively late stage, after lactic acid, protease and flavour production by the L.lactis organism has already taken place.

By keeping the productive strain pure, either in the starter culture or in the milk or other medium, uniformity of production can be improved.

The bacteriocin may also be used to kill selectively strains of lactic acid producing bacteria in beer and distillery fermentations, since these are attributed in the literature to be the major cause of spoilage in unpasteurised beers and give rise to the greatest proportion of infections during fermentation.

Other bacterial species than clostridia or L.lactis may also present problems in the spoiling of foods during processing or manufacture. For example, B. cereus present in foods such as rice may lead to food poisoning.

Strains of clostridium in particular are known to cause a problem in contaminating food manufacturing processes and may lead for example to the spoiling of cheese. At present, clostridial contamination is dealt with either by treatment with nitrates, which are presently recognised to have a number of disadvantage, notably from the toxicity point of view or using lysozyme which is not generally effective. The use of bacteriocin according to the invention thus presents a considerable advance over such prior art methods. This is an area of considerable commercial and economic

importance and a further aspect of the invention provides use of the novel bacteriocin of the invention in selectively killing undesired or contaminating strains of bacteria, eg. lactic acid bacteria, Clostridia, or Bacillus species in microbiological processes such as fermentation (eg. ethanol fermentation) or food manufacturing processes, eg. in dairy processes such as cheese or yoghurt production.

The invention also includes starter cultures of microorganisms containing the bacteriocin as an inhibitor of contaminating bacterial eg. lactococcus or clostridia species. Such microorganisms may, for example, be strains of L.lactis resistant to the bacteriocin eg. the producing organism, so that only unwanted microorganisms are removed from the starter culture, or yeasts of use in beer or distillery fermentations. Such starter cultures will normally be in lyophilised form.

Further uses of the novel bacteriocin include the production of cell wall preparations or for liberation of nucleic acid material.

The novel bacteriocin of the invention may be isolated from cultures of Lactococcus lactis strain LMG 2081 by fractionation of the growth medium whereby fractions enriched in the bacteriocin are collected. Known fractionation techniques may be applied to obtain the bacteriocin in electrophoretic purity. Thus for example the organism may be grown in a suitable culture medium eg. M17 broth (oxoid) and the supernatent subjected to fractional precipitation eg. with ammonium sulphate followed by chromatography eg. a combination of ion exchange, hydrophobic and reverse phase chromatography.

We have found in particular that the novel bacteriocin may be purified to homogeneity by a simple 4 step purification procedure which includes ammonium sulphate precipitation, followed by cation exchange,

octyl sepharose and reverse phase chromatography. Using this procedure up to a 7000-fold increase in specific activity may be obtained.

A further aspect of the invention thus includes a method of isolation of bacteriocin according to the invention, wherein a culture of a microorganism expressing said bacteriocin is subjected to fractionation whereby fractions enriched in said bacteriocin are collected. Preferably, in such a method, the expressing organism is L.lactis strain LMG 2081.

Nucleic acid molecules comprising a nucleotide sequence encoding the novel bacteriocin or its component peptides and/or its corresponding immunity factor (which provides resistance against self-destruction in the producing strain) respectively form further aspects of the invention.

The region of the L. Lactis LMG 2081 genome coding for the novel bacteriocin of the invention and its immunity factor has been identified and sequenced. In particular, we have identified and cloned an operon which includes genes coding for the bacteriocin component peptides in their pro form as well as for a further protein believed to be the immunity factor providing resistance against self-destruction by the bacteriocin. The sequence of the operon is shown in Figure 1 (SEQ ID NO: 5), which also shows the corresponding predicted amino acid sequence (SEQ ID NO: 1). The putative promoter region and ribosome binding site are indicated. The gene (designated lag a) coding for the pro-sequence of the α peptide (designated lag A) appears to run from nucleotide 536 and a vertical arrow indicates where the peptide is cleaved between the amino acids corresponding to nucleotides 580-581 to give the mature α peptide.

Translation of the β pro-sequence (designated lag b for the gene and lag B for the pro-peptide) starts at

nucleotide 746 and again a vertical arrow indicates where cleavage occurs at the amino acids corresponding to between nucleotides 820-821 to give the mature β peptide. Downstream of the above-mentioned sequence is a sequence comprising a single long open reading frame, starting at nucleotide 1027 and encoding a putative polypeptide of 110 amino acids, starting with LFNN and ending with LFIR, believed to be the immunity factor (designated lag C).

The genes designated lag d and lag e are believed to code for proteins (designated lag D and lag E) that are involved in the secretion of the mature α and β peptides; the lag d and lag e sequences show homology to genes with such functions in other systems.

Accordingly, a further aspect of the invention provides a nucleic acid molecule comprising a nucleotide sequence which encodes a bacteriocin, its component peptides and/or its corresponding immunity factor, or a fragment thereof, substantially corresponding to all or a portion of the nucleotide sequence as shown in Figure 1 (SEQ ID NO: 5) or a sequence which is degenerate or substantially homologous with or which hybridises with any such sequence.

Such nucleic acid molecules may be single or double stranded DNA, cDNA or RNA, preferably DNA and include degenerate, substantially homologous, and hybridising sequences which are capable of coding for the bacteriocin concerned. "Substantially homologous" as used herein includes sequences displaying at least 60%, preferably at least 70% or 80% sequence homology and also functionally-equivalent allelic variants and related sequences modified by single or multiple base substitution, addition and/or deletion. By "functionally-equivalent" is meant nucleotide sequences encoding polypeptides having essentially equivalent bacteriocin activity.

Nucleic acid sequences which hybridise with the

sequence shown in Figure 1 (SEQ ID NO: 5) or any substantially homologous or functionally-equivalent sequences as defined above are also included within the scope of the invention. "Hybridisation" as used herein defines those sequences binding under non-stringent conditions (eg. 6 x SSC 50% formamide at room temperature) and washed under conditions of low stringency (eg. 2 x SSC, room temperature, more preferably 2 x SSC, 42°C) or conditions of higher stringency (eg. 2 x SSC, 65°C) (where SSC = 0.15M Nacl, 0.015M sodium citrate, PH7.2). Generally speaking, sequences which hybridise under conditions of high stringency are included within the scope of the invention, as are sequences which, but for the degeneracy of the code, would hybridise under high stringency conditions.

Derivative nucleotide sequences capable of encoding bacteriocin or bacteriocin derivatives according to the invention may be obtained by using conventional methods well known in the art. These include site-directed mutagenesis, random mutagenesis, or enzymatic cleavage and/or ligation of nucleic acids.

Bacteriocin according to the invention may be prepared in recombinant form by expression in a host cell containing a recombinant DNA molecule which comprises a nucleotide sequence as broadly defined above, operatively linked to an expression control sequence, or a recombinant DNA cloning vehicle or vector containing such a recombinant DNA molecule.

Appropriate recombinant DNA techniques are well known in the art and are described for example by Sambrook et al., 1989, (Molecular Cloning, a laboratory manual, 2nd Edition, Cold Spring Harbour Press).

The bacteriocin so expressed may be a fusion polypeptide comprising all or a portion of the bacteriocin according to the invention and an additional polypeptide coded for by the DNA of the recombinant

molecule fused thereto. This may for example by β-galactosidase, glutathione-S-transferase, or any of the other polypeptides commonly employed in fusion proteins in the art.

Other aspects of the invention thus include cloning and expression vectors containing nucleic acid molecules according to the invention coding for the bacteriocin and/or for the immunity factor. Expression vectors appropriate to L. lactis are preferred. Such expression vectors include appropriate control sequences such as for example translational (eg. start and stop codes) and transcriptional control elements (eg. promoter-operator regions, ribosomal binding sites, termination stop sequences) linked in matching reading frame with the nucleic acid molecules of the invention.

The invention also includes transformed or transfected prokaryotic or eukaryotic host cells, or transgenic organisms containing a nucleic acid molecule according to the invention as defined above. Such host cells may for example be transformed strains of lactic acid bacteria eg. strains of L. lactis.

Also included within the scope of the invention are methods for preparing the polypeptides of the invention comprising culturing a host cell containing a nucleic acid molecule as defined above under conditions whereby said polypeptide is expressed and recovering said polypeptide then produced.

The nucleic acid coding for the new bacteriocin and/or immunity factor may be incorporated into any convenient cloning vector for amplification and into an expression vector for transformation of host microorganisms such as L. lactis, for example cloning vector pIL253 (Simon and Chopin, Biochimie 70, 1988, 59-566). Growth under suitable culture conditions will provide the bacteriocin in the growth medium, from which it can be isolated by the techniques described above.

Furthermore, host cells such as strains of lactic

acid bacteria eg. L. lactis may be transformed with multiple copies of a plasmid or other vector containing the required nucleic acid sequence to provide an improved strain giving rise to enhanced production of the bacteriocin. Such improved strains may provide more rapid killing and hence accelerated cheese ripening when used in cheese manufacture. In particular, the strain of L.lactis which produces the bacteriocin may be provided with such multiple copies of the vector; this will thus be able to proliferate without premature destruction by the bacteriocin.

The new bacteriocin may also be prepared by chemical synthesis, for example using solid phase synthesis, advantageously using a polypeptide synthesis apparatus, as commercially available. In such a synthesis, active side chain groupings (e.g. amino or carboxyl groups) of the respective amino acids will be protected and the final step will be deprotection and/or removal from the inert support to which the polypeptide is attached during synthesis.

In building up the peptide chains, one can in principle start either at the C-terminal or the N-terminal although only the C-terminal starting procedure is in common use.

Thus, one can start at the C-terminal by reaction of a suitable derivative of, for example histidine with a suitable protected derivative of leucine. The histidine derivative will have a free α-amino group while the other reactant will have either a free or activated carboxyl group and a protected amino group. After coupling, the intermediate may be purified for example by chromatography, and then selectively N-deprotected to permit addition of a further N-protected and free or activated amino acid residue. This procedure is continued until the required amino acid sequence is completed.

Carboxylic acid activating substituents which may,

for example, be employed include symmetrical or mixed anhydrides, or activated esters such as for example p-nitrophenyl ester, 2,4,5,trichlorophenyl- ester, N-hydroxybenzotriazole ester (OBt), N-hydroxy-succinimidylester (OSu) or pentafluorophenylester (OPFP).

The coupling of free amino and carboxyl groups may, for example, be effected using dicyclohexylcarbodi-imide (DCC). Another coupling agent which may, for example, be employed is N-ethoxycarbonyl-2-ethoxy-1,2-dihydro-quinoline (EEDQ).

In general it is convenient to effect the coupling reactions at low temperatures, for example, -20°C up to ambient temperature, conveniently in a suitable solvent system, for example, tetrahydro- furan, dioxan, dimethylformamide, methylene chloride or a mixture of these solvents.

It may be more convenient to carry out the synthesis on a solid phase resin support. Chloro-methylated polystyrene (cross-linked with 1% divinyl benzene) is one useful type of support; in this case the synthesis will start the C-terminal, for example by coupling N-protected histidine to the support.

A number of suitable solid phase techniques are described by Eric Atherton, Christopher J. Logan, and Robert C. Sheppard, J. Chem. Soc. Perkin I, 538-46 (1981); James P. Tam, Foe S. Tjoeng, and R. B, Merrifield J. Am. Chem. Soc. 102, 6117-27 (1980); James P. Tam, Richard D. Dimarchi and R. B. Merrifield Int. J. Peptide Protein Res 16 412-25 (1980); Manfred Mutter and Dieter Bellof, Helvetica Chimica Acta 67 2009-16 (1984).

A wide choice of protecting groups for amino acids are known and are exemplified in Schröder, E., and Lübke, K., The Peptides, Vols. 1 and 2, Academic Press, New York and London, 1965 and 1966; Pettit, G.R., Synthetic Peptides, Vols. 1-4, Van Nostrand, Reinhold, New York 1970, 1971, 1975 and 1976; Houben-Weyl,

Methoden der Organischen Chemie, Synthese von Peptiden, Band 15, Georg Thieme Verlag Stuttgart, NY, 1983; The Peptides, Analysis, synthesis, biology 1-7, Ed: Erhard Gross, Johannes Meienhofer, Academic Press, NY, San Fransisco, London; Solid phase peptide synthesis 2nd ed., John M. Stewart, Janis D. Young, Pierce Chemical Company.

Thus, for example amine protecting groups which may be employed include protecting groups which may be employed include protecting groups such as carbobenzoxy (Z-), t-butoxycarbonyl (Boc-), 4-methoxy-2,3,6-trimethyl-benzene sulphonyl (Mtr-), and 9-fluorenylmethoxycarbonyl (Fmoc-). It will be appreciated that when the peptide is built up from the C-terminal end, an amine protecting group will be present on the α-amino group of each new residue added and will need to be removed selectively prior to the next coupling step. One particularly useful group for such temporary amine protection is the Fmoc group which can be removed selectively by treatment with piperidine in an organic solvent.

Carboxyl protecting groups which may, for example be employed include readily cleaved ester groups such as benzyl (-OBZl), p-nitrobenzyl (-ONB), or t-butyl (-tOBu) as well as the coupling on solid supports, for example methyl groups linked to polystyrene.

It will be appreciated that a wide range of other such groups exists as, for example, detailed in the above-mentioned literature references, and the use of all such groups in the hereinbefore described processes fall within the scope of the present invention.

A wide range of procedures exists for removing amine- and carboxyl-protecting groups. These must, however, be consistent with the synthetic strategy employed. The side chain protecting groups must be stable to the conditions used to remove the temporary α-amino protecting groups prior to the next coupling

step.

Amine protecting groups such as Boc and carboxyl protecting groups such as tOBu may be removed simultaneously by acid treatment, for example with trifluoro acetic acid.

A further aspect of the invention provides a process for the preparation of bacteriocin polypeptides according to the invention in which a corresponding protected or immobilised polypeptide is subjected to deprotection or removal from a solid support.

The invention will now be described in more detail in the following non-limiting Example with reference to the following Figures in which:-

Figure 2 shows reverse phase chromatography of lactococcin G (fraction IV). (A) The optical density profile (----------) and propanol gradient (---- --- --). (B) Bacteriocin activity without complementation (....•....), with complementation with α_1 (-----•-----), and with complementation with β (----O----). The amount applied on the column represents that obtained from approximately 2 liter culture;

Figure 3 shows reverse phase chromatography of (A) α_2 (B) β, and (C) α_1. The optical density profile (----------) and propanol gradient (---- --- --). The bacteriocin activity without complementation (....•....) and with complementation (----O----) with (B) α_1, or with (A and C) β . The amount applied on the column represents that obtained from approximately 2 liter culture;

Figure 4 shows the amino acid sequence of α_1, α_2 and β (SEQ ID NOS: 2 and 3). N and C indicates the, respectively, N- and C- terminal ends of the peptide;

Figure 5 shows the amount of α_1 and β which in combination inhibited growth of the indicator strain by 50%;

Figure 6 shows an Edmundson α-helical wheel representation of the amphiphilical region in (A) α_1 and

(B) β. For α_1 the amphiphilical region shown starts with residue number 3 and ends with number 27, for β it starts with residue number 8 and ends with number 25. The shaded areas indicate non-polar residues, whereas the light areas indicate polar residues.

Example 1

MATERIALS AND METHODS

Bacterial strains and media

The bacteriocin producer was strain Lactococcus lactis LMG 2081, obtained from J. Narvhus, Agricultural University, As, Norway. The indicator organism used in the bacteriocin assay was lactococcus lactis subsp. lactis IL 1403 (Chopin, et al., Plasmid, 11: 260-263, 1984.) Both strains were grown at 30°C in M17 broth (Oxoid) without lactose, but supplemented with 0.4% (wt/vol) glucose. The M17 broth was also supplemented with Tween 80 to a final concentration of 0.1% (vol/vol) when culturing strain LMG 2081 for the production of bacteriocin.

Bacteriocin assay

The bacteriocin was quantified in a microtiter plate assay system (Gels, et al., Appl. Environ. Microbiol. 45: 205-211, 1983). Each well of the microtiter plate contained 200 µl of M17 broth (supplemented with 0.4% (wt/vol) glucose and 0.1% (vol/vol) Tween 80), bacteriocin fractions at two-fold dilutions, and the indicator organism, Lactococcus lactis subsp. lactis IL 1403 (A_{600} = 0.1). The microtiter plate cultures were incubated 3 hours at 30°C, after which growth inhibition of the indicator organism was measured spectrophotometrically at 500 nm b using a Dynatech Microplate Reader. One bacteriocin

unit (BU) was defined as the amount of bacteriocin which inhibited growth of the indicator organism by 50% (50% of the turbidity of the control culture without bacteriocin).

Bacteriocin purification

All the purification steps were performed at room temperature, and all the chromatographic equipment, was obtained from Pharmacia-LKB Biotechnology (Uppsala, Sweden). The bacteriocin was purified from 2-liter cultures of Lactococcus lactis LMG 2081 grown to the late exponential/early stationary phase. The cells were removed by centrifugation at 4,000 x g for 15 min at 4°C, and 400 g of ammonium sulfate per liter culture supernatant was added. The protein precipitate was pelleted by centrifugation at 7,000 x g for 20 min and solubilised in 20 mM sodium phosphate buffer, pH 5.7 (buffer A, 250 ml per 2 liter culture) (fraction I). Fraction I was applied to a flow rate of about 10 ml/min to a 7 ml S-Sepharose Fast Flow cation exchange column equilibrated with buffer A. After subsequently washing the column with 20 ml of buffer A, the bacteriocin was eluted from the column with 40 ml 1 M NaCl in buffer A (fraction II). Ammonium sulfate was added to fraction II to a final concentration of 10% (wt/vol), after which the fraction was applied at a flow rate of about 4 ml/min to a 2 ml Octyl-Sepharose CL-4B column equilibrated with 10% (wt/vol) ammonium sulfate in buffer A. The column was then washed with 8 ml of buffer A, after which the bacteriocin activity was eluted from the column with 10 ml 70% (vol/vol) ethanol and 30% buffer A (fraction III). Fraction III was diluted to 50 ml with H_2O containing 0.1% (vol/vol) trifluoroacetic acid (TFA) and subsequently applied to a C_2/C_{10} reverse-phase column, PepRPC HR 5/5, equilibrated with 2-propanol/H_2O (10 : 90), containing 0.1% TFA. The bacteriocin was eluted with a linear gradient ranging

from 30 to 50% 2-propanol containing 0.1% TFA (fraction IV). The Bacteriocin peptides eluting from the reverse phase column (fraction IV) were in some cases diluted 4-5-fold with H_2O containing 0.1% TFA and rechromatographed on the reverse phase column. Purified bacteriocin was stored in 50-60% 2-propanol and or ethanol containing 0.1% TFA at -20°C.

Mass spectroscopy analysis

Mass spectroscoopy analysis of peptides was performed using the Biolon Mass Analyser (Applied Biosystem, Sweden) as described earlier by Sorensen et al., Biomed. Environ. Mass Spectrom. 19: 713-720, 1990. Peptide fractions were dissolved in 50-100 µl TFA containing 20% acetonitrile. From each fraction, 5 µl were loaded to a target and data accumulated for 10 min at 16 kV.

Amino acid sequencing

The amino acid sequence was determined by Edman degradation using an Applied Biosystems (Foster City, Calif.) 477A automatic sequencer with an online 120A phenylthiohydantoin amino acid analyser.

RESULTS

Purification of bacteriocin

Initial screening showed that Lactococcus lactis LMG 2081 produced antagonistic activity towards various LAB and a number of different Clostridia. This strain produced bacteriocin constitutively during growth, although maxium activity was found in the culture medium at the very end of the exponential or early stationary phase of growth. The activity started to decrease after a few hours in the stationary phase. The stability of the bacteriocin in the culture depended on the bacteriocin producing strain. With other strains which

produced the same bacteriocin, maximum activity was found in the middle of the exponential phase, whereas little if any activity was found towards the end of this growth phase. This may be due to the cell envelope-associated preteinase produced by these strains, as this proteinase rapidly degrades the bacteriocin when present in the culture media.

The purification scheme developed for isolating the bacteriocin for sequencing is shown in Table 1.

TABLE 1. Purification of Lactococcin G

Fraction	Vol (ml)	Total A_{280}[a]	Total activity (BU)	Sp act[b]	Increase sp act[b]	Yield (%)
Culture supernatant	2000	67,000	30×10^6	450	1	100
Ammonium sulfate precipitation (fraction I)	250	1,050	17×10^6	16,000	35	57
Binding to cation exchanger (fraction II)	40	5.8	6×10^6	1×10^6	2,200	20
Binding to Octyl Sepharose (fraction III)	10	2.5	6×10^6	2.5×10^6	5,600	20
Reverse phase chromatography (fraction IV)	1	1.5	5×10^6	3×10^6	6,700	17

[a] Total A_{280} is the absorbance at 280 mm multiplied by the volume in ml.

[b] Specific activity is bacteriocin units (BU) divided by the absorbance at 280 nm.

Tween 80 (final concentration of 0.1% (vol/vol) was added to the culture medium before ammonium sulfate precipitation in order to obtain binding of the bacteriocin to the cation exchanger for bacteriocin activity, as this increased the sensitivity of the assay 2-10 fold. The bacteriocin was concentrated 8-fold from the culture media by ammonium sulfate precipitation. This resulted in a 30 to 40-fold increase in the specific activity and a recovery of about 60% of the activity (Table 1, fraction 1). By subsequently binding the bacteriocin in fraction 1 to a cation exchanger and eluting it with 1 M NaCl, an increase in the specific activity of more than 2,000 was obtained (Table 1, fraction II). From this stage and on the yield remained at about 20% (Table 1). The specific activity increased 5,000-6,000-fold after binding the bacteriocin in fraction II to Octyl-Sepharose and eluting it with 70% ethanol (Table 1, fraction III). When fraction III was applied to the reverse phase column and eluted with a steep propanol gradient (6%/ml), the bacteriocin activity coeluted with an absorbance peak at about 40% propanol (results not shown). This absorbance peak, however, did not appear to be entirely homogeneous, as two shoulders could be discerned on both sides of the main peak.

Bacteriocin activity depends on the complementary action of two peptides

Upon rechromatography of fraction IV, this time eluting the bacteriocin activity using a shallow propanol gradient (0.5%/ml), 4 absorbance peaks were obtained (Fig. 2). The last 3 of these peaks were termed α_2 ß and α_1 in the order of which they eluted together with the α_1 absorbance peak, but the total activity was greatly reduced compared to that which was applied to the column (Fig. 2). However, upon adding an aliquot of the fraction containing α_1 to each of the

column fractions, there was a complete recovery of bacteriocin activity in the fraction containing β (Fig. 1). Similarly there was a complete recovery of bacteriocin activity in the fraction containing α_1, and to a lesser extent in the fraction containing α_2, when an aliquot of ß was added to each fraction (Fig. 2).

Each peptide α, α_2 and ß was purified to homogeneity by rechromatography on the reverse phase column (Fig. 3). Whereas relatively little activity was seen when each peptide was assayed for bacteriocin activity alone, the activity was recovered when the ß peptide was complemented with the α peptide, and to a lesser extent with the α peptide (Fig. 3). No additional increase in the bacteriocin activity was seen upon adding the α_2 peptide to fractions containing both the ß and α_1 peptides. Thus the complementary action of the two peptides, an α and a ß peptide appeared to be necessary to obtain bacteriocin activity.

A small amount of ß which presumably had not been entirely separated from α_1 on the previous reverse phase column, was detected upon rechromatography of α_1 (Fig. 3 C). A small optical density peak apparently due to α_2 as well as a minor peak eluting ahead of α_2 - similarly to the optical density peak which eluted slightly ahead of α_2 in Fig. 2 - were also detected (Fig. 3 C). It is unlikely that the presence of these two latter peaks was due to incomplete separation from α_1 on the previous reverse phase column, since they did not contaminate the ß preparation to the same extent (Fig. 3 B). Upon rechromatography of purified α_1 after storage in 50% propanol for 3 months at -20°C, 30-40% of the peptides eluted as expected for α_1, whereas 30-40% eluted similarly to α_2 in Fig 2. As much as 10% of purified α_1 eluted similarly to α_2 upon rechromatography after storage for 24 h. This suggests that α_2 and the component which eluted ahead of α_2 were derived from α_1.

Mass spectroscopy analysis of the α and ß peptides

In order to determine the molecular weights of α_1 and ß and confirm their purity after the final reverse phase chromatography step (Fig. 3), these peptides were analyzed by plasma disorption mass spectroscopy. Single peaks were observed with molecular weights of 4376 and 4109 for α and ß, respectively.

Amino acid sequence of the α and ß peptides

The complete amino acid sequence of the α_1 and α_2 (39 amino acid residues) and ß (35 amino acid residues) peptides (SEQ ID NOS: 2 and 3) are shown in Fig. 4. It appears from the sequences that α_2 is identical to α_1 (Fig. 4), consistent with the apparent formation of α_2 peptides upon rechromatography of α_1 on the reverse phase column.

Relative amounts of α and ß to obtain bacteriocin activity

The concentrations of α_1 and ß which in combination inhibited growth of the indicator organism by 50% were determined, and the results were plotted as an isobologram (Fig. 5). When α_1 was in excess (greater than 1.3 nM, 0.25 pmoles/well), the presence of ß at a concentration of 0.02 nM (0.04 pmoles/well), resulted in 50% growth inhibition (Fig. 5). Similarly, with an excess of ß (greater than 0.45 nM (0.03 pmoles/well), the presence of α_1 at a concentration of about 0.15 nM (0.03 pmoles/well) resulted in a 50% growth inhibition (Fig. 5). Thus in order to obtain 50% growth inhibition in the presence of an excess of the complementary peptide, 7-8-fold more α_1 than ß was needed. When neither α_1 nor ß was in excess, the concentrations which resulted in 50% growth inhibition were 0.3 nM (0.06 pmoles/well) for α_1 and 0.04 nM (0.008 pmoles/well) for ß (Fig. 5). Again there was 7-8-fold more of α_1 than ß. The concentrations which inhibited growth by 50%

appeared to be invariant to the number of target cells present (within a 30fold range in the cell number), as the same concentrations of α_1 and ß resulted in 50% growth-inhibition irrespective of whether the cell density was such that the A was 0.01 or 0.3.

Summary

Three optical density peaks associated with bacteriocin activity were obtained upon reverse phase chromatography in the final purification step. The peptides associated with the 3 optical density peaks were termed α_1, α_2 and ß.

The bacteriocin activity was due to the complementary action of an α and the ß peptide. In combination with the ß peptide, α_1 gave a mugh higher bacteriocin activity than α_2. Upon rechromatography of purified α_1 on a reverse phase column, some of it eluted as expected for α_2. This suggests that α_1 and α_2 may in fact be the same peptide, but that they differ in their configuration in a manner which results in α_2 having a slightly lower affinity to the reverse phase column and reduced activity when combined with ß than α_1. This view was supported by the amino acid sequencing data. The α_2 peptide was sequenced and this sequence appeared to be identical to the corresponding sequence of α_1.

As judged by amino acid sequencing, α_1 contained 39 amino acid residues and its molecular weight should be 4346. A molecular weight of 4376 was obtained by mass spectrometry indicating that the peptide is not grossly modified. Judging from its sequence, ß contains 35 amino acid residues and its molecular weight should be 4110. This is in good agreement with the molecular weight of 4109 obtained by mass spectrometry, indicating that this peptide is not modified. From the amino acid sequence, the isoelectric point and extinction coefficient of α_1 were calculated to be 10.9 and 1.3 x 10^4 M^{-1} cm^{-1}, respectively. For the ß peptide the

isoelectric point and extinction coefficient were calculated to be 10.4 and 2.4 x 10^4 M^{-1} cm^{-1} respectively.

The amino acid sequence of both α and ß is such that these peptides are likely to be pore-forming toxins that create cell membrane channels through a "barrel-stave" mechanism, and thus produce an ionic imbalance in the cell (See Ojcius et al, TIBS, 16 225-229, 1991). A region in α_1 starting with amino acid residue number 3 and ending with residue number 27 may form an amphiphilic α-helix, as is evidence when this sequence is displayed on an Edmundson α-helical wheel (Fig. 6A). The polar amino acids are found almost completely on one side of the α-helix, whereas the nonpolar residues are found on the opposite side of the helix (Fig. 6A). The amphiphilic distribution of the amino acids in this region is nearly perfect, the only exception being glycine (residue number 9) which appears on the hydrophobic side (Fig. 6A). However, glycine may be considered to be relatively neutral with respect to its hydrophilic/hydrophobic character. Moreover, the substitution of one amino acid by one of an opposite hydrophobicity may not represent an intolerable disruption of a peptide's amphiphilic character (see Ojcius, Supra). The 25 amino acids long amphiphilic region in α_1 may allow peptide-monomers to oligomerize into membrane-spanning pores in such a manner that the non-polar side of the α-helix faces the membrane lipids, whereas the polar side faces towards the center of the pore (see Ojcius (Supra) and Lear et al, Science 240:1177-1181, 1988). The amphiphilic region in α_1 should be long enough to span a membrane, as a minimum of about 20 residues are needed to form a membrane-spanning α-helix. Ten of the 12 C-terminal amino acid residues, starting with position 28 and ending with 39, in α_1 are polar, of which the last 5 are basic (Arg-Lys-Lys-Lys-His-COOH). In this connection it is interesting

to note that lactococcin A and lactocin S, bacteriocins produced by Lactococcus lactis subsp cremoris and Lactobacillus sake. respectively, also contain a basic C-terminus, the last two C-terminal amino acids being histidine for both of these bacteriocins, (Holo (Supra), Mørvedt (Supra)). It seems likely that the 11 amino acid residues long polar C-terminal region in α_1 does not penetrate the membrane. One may speculate that its function might be to recognise bacteriocin-binding sites on the target cells and thereby create a local high concentration of bacteriocin monomers on the outside of the cell membrance. This high concentration near the membrane could then induce oligomerization of the monomers into transmembrane pores. Another possible function of the polar C-terminal region might be to stabilize a correct peptide-configuration in a hydrophilic environment. The first two N-terminal amino acid residues in α_1 are polar, and may possibly be the part of the pore which is located inside the cell.

The situation is similar for the ß-peptide. An amphiphilic α-helix may be formed in the region starting with amino acid residue number 8 and ending with residue number 25 (Fig 6 B). In this region there are two exceptions to a perfect amphiphilic amino acid distribution: glycine (residue 22) which appears on the hydrophobic side and isoleucine (residue 24) which appears on the hydrophilic side. The former should not be a major problem, due to the neutral character of glycine. One would expect that proline at position 11 would cause a break or bend in the α-helix structure of the amphiphilic region. The region spans 18 amino acid residues, which may be somewhat less than required to span the cell membrane. However, in front of the amphiphilic region there are 5 nonpolar amino acids starting with residue number 3 and ending with number 7 which presumably also will be part of the transmembrane region. The hydrophilic amino acid, lysine as found at

positions 1 and 2 at the N-terminus; and only 2 of 10-residues starting with position 26, in the C-terminal part of the molecule are hydrophobic. Similar to α_1, one might expect that these regions will be located outside the membrane, the long polar C-terminal region being on the cell's outside. Although other lactic acid bacteria-produced bacteriocins that have been sequenced do not appear to have such a marked amphiphilic distribution of amino acids as α and ß such a distribution is also seen in the C-terminal part of lactococcin A and Lactocin S. There is evidence that lactococcin A, permeabilizes target cell membranes (Van Belkum (Supra) as also appears to be the case for nisin (Sahl et al., Arch Microbiol 149: 120-124, 1987).

The concentrations of α_1 and ß which inhibited growth of the indicator cells by 50% were respectively 0.15 and 0.02 nM when the complementing peptide was present in excess. When neither was in excess the concentrations were, respectively, 0.3 and 0.04 nM. Thus 7-8-fold more of α_1 than ß was needed. If the two peptides associate to the target cells with equal efficiency, this ration may reflect that α_1 and ß interact in an approximately 8 to 1 ratio, for instance in pore formation. The presence of approximately 40 ß molecules per target cell together with an excess of α_1 is enough to induce 50% growth inhibition. The number of ß molecules that interact with a target cell may possibly be even smaller, since the concentration of bacteriocin which inhibited growth by 50% appeared to be invariant to the number of target cells present within a 30-fold range.

Example 2

Cloning and sequencing of the bacteriocin (lactococcin G (LcnG)) was carried out as follows.

Chromosomal DNA was purified from Lactococcus lactis LMG 2081 according to standard methods, and digested with SpeI. SpeI fragments were fractionated by agarose gel electrophoresis, and DNA fragments of 3-7 kbp were isolated using Magic Minipreps (Promega). A sub-genomic library was constructed by ligating the fragments with SpeI digested and phosphatase treated lambda ZAP II arms (stratagene). The clone containing the bacteriocin (LcnG) structural gene and immunity gene was isolated by screening the library with a degenerate oligonucleotide probe deduced from the amino acid sequence of LcnG (Nissen-Meyer et al.). The hybridization was carried out according to standard protocols (Molecular cloning, Eds; Sambrook, et al., supra). Sequences were determined by the chain termination method (Sanger et al.) using sequenase (United States Biochemical Corp.). The sequence obtained is shown in Figure 1 (SEQ ID NO: 5).

CLAIMS

1. A polypeptide having or including an amino acid sequence substantially corresponding to all or a portion of the amino acid sequence set out in Figure 1 (SEQ ID NO: 1) and derivatives and fragments thereof having bacteriocin and/or bacteriocin immunity activity.

2. A polypeptide as claimed in claim 1 having or including the amino acid sequence

α_1 and α_2 (SEQ ID NO: 2):

N Gly Thr Trp Asp Asp Ile Gly Gln Gly
Ile Gly Arg Val Ala Tyr Trp Val Gly
Lys Ala Met Gly Asn Met Ser Asp Val
Asn Gln Ala Ser Arg Ile Asn Arg Lys
Lys Lys His C

and/or

β (SEQ ID NO: 3):

N Lys Lys Trp Gly Trp Leu Ala Trp Val
Asp Pro Ala Tyr Glu Phe Ile Lys Gly
Phe Gly Lys Gly Ala Ile Lys Glu Gly
Asn Lys Asp Lys Trp Lys Asn Ile C

and derivatives and fragments thereof having bacteriocin activity.

3. A polypeptide having or including the amino acid sequence

Leu Phe Asn Asn Ile Val Val Phe Ile
Asn Phe Leu Ser Phe Val Phe Ile Leu
Val Gly Val Asp Ile Lys Tyr Asn Asp
Asn Arg Ile Lys Ile Val His Val Thr
Phe Phe Ile Ser Phe Ile Leu Val Met

Leu	Thr	Ser	Leu	Ile	Ser	His	Asn	Ser
Ile	Ala	Tyr	Ser	Leu	Ser	Gln	Ile	Leu
Glu	Ile	Leu	Cys	Ile	Ile	Cys	Ile	Leu
Leu	Leu	Phe	Tyr	Ile	Leu	Lys	Lys	Thr
Asn	Ser	Leu	Ser	Asn	Arg	Ala	Asn	Val
Val	Phe	Ile	Ile	Phe	Ile	Val	Thr	Gln
Val	Ile	Ile	Ile	Ile	Asn	Gln	Leu	Phe
Ile	Arg							

(SEQ ID NO: 4) and derivatives and fragments thereof having bacteriocin immunity factor activity.

4. A bacteriocin comprising polypeptides α and β or fragments or derivatives thereof as defined in claim 2.

5. A bacteriocin as claimed in claim 4 comprising polypeptides α and β, or fragments or derivatives thereof, in a ratio of 5-10 to 1 respectively.

6. A bacteriocin as claimed in claim 5 comprising polypeptides α and β, or fragments or derivatives thereof, in a ratio of 8 to 1 respectively.

7. A composition comprising a bacteriocin as claimed in any one of claims 4 to 6 and/or a bacteriocin immunity factor as claimed in claim 3 together with at least one of a carrier, and/or diluent, or excipient.

8. Use of a bacteriocin as claimed in any one of claims 4 to 6 in selectively killing undesired or contaminating strains of bacteria in microbiological or food manufacturing processes.

9. A starter culture of microorganisms for use in a microbiological process, comprising a bacteriocin as claimed in any one of claims 4 to 6, said microorganisms being resistant to said bacteriocin.

10. A method of cheese or yoghurt production in which a bacteriocin as claimed in any one of claims 4 to 6 is added to effect lysis of lactic acid bacteria.

11. A method of isolation of a bacteriocin and/or a bacteriocin immunity factor as claimed in any one of claims 3 to 6 wherein a culture of a microorganism expressing said bacteriocin and/or immunity factor is subjected to fractionation whereby fractions enriched in said bacteriocin and/or immunity factor are collected.

12. A method as claimed in claim 11 wherein the microorganism is Lactococcus lactis strain LMG 2081.

13. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide or its derivatives or fragments as claimed in any one of claims 1 to 3.

14. A nucleic acid molecule comprising a nucleotide sequence which encodes a bacteriocin, its component peptides and/or its corresponding immunity factor, or a fragment thereof, substantially corresponding to all or a portion of the nucleotide sequence as shown in Figure 1 (SEQ ID NO: 5) or a sequence which is degenerate or substantially homologous with or which hybridises with any such sequence.

15. An expression or cloning vector comprising a nucleic acid molecule as claimed in claim 13 or claim 14.

16. A host cell or transgenic organism containing a nucleic acid molecule as claimed in claim 13 or claim 14.

17. A host cell as claimed in claim 16 being a lactic acid bacterium.

18. A method for preparing a polypeptide, derivative or fragment as claimed in claim 1, comprising culturing a host cell as defined in claim 16 or claim 17 under conditions whereby said polypeptide is expressed and recovering said polypeptide, derivative or fragment thus produced.

19. A process for the preparation of bacteriocin and/or bacteriocin immunity factor polypeptides as claimed in any one of claims 1 to 3 in which a corresponding protected or immobilised polypeptide is subjected to deprotection or removal from a solid support.

FIG. 1 (1/6)

1 TACTCAAAAATTATGGGGAATTTGACTATGACTACGTTAGTAAACTAATTGTAGAAGAAAAAATAACAGAAAATACCGTCA
82 AGCGTCGAGCAAGTACGGTCTGTTCATGGATAAATTGGATATTATCGGTTGTGGAGTAGATATTTTAATATTGAAAAGTAT
163 ATTGTGTTGACTATTCGAACAATAATAAGATTTATAAATAAATTTATTAAATAAAAAATCACTGGCAATAATATCTAGTGA
244 TTTTTTATTTAGTGAATTTTGATATATAAATATGATTTAATACTTTCGTAGAGGTTAAAGAATAGTAACTATGTTGCCTAT
325 ACAAAAATTCAAGGAGATGTCTCCACAAATATGGAAAGGCGCAAAAAATATCTCTTTATCAAGCATTTATTTTAATTATTT
406 CCATCGCAATAGTAAGATTGGCACTTAATAAAAAATAATACTCGATTTTTAGAATAAAATTAAATATGATATTGATTTATG
-35
lagA
487 TTACTAAATATGATATAATATTCTAATAATAAATAAAAGGAGAATTTTTATGAAAGAATTATCAGAAAAAGAATTACGAGA
-10 RBS M K E L S E K E L R E
568 ATGCGTTGGCGGTGGAACTTGGGATGATATTGGTCAAGGAATAGGAAGAGTCGCTTATTGGGTTGGAAAAGCCATGGGAAA
C V G G G T W D D I G Q G I G R V A Y W V G K A M G N
649 TATGAGCGATGTTAATCAAGCTTCTAGAATTAATAGAAAAAAGAAACACTAATTTATAAGACTTATAATATTTAGCAAAAT
M S D V N Q A S R I N R K K K H *
lagB
730 ATGGAGAGTGAAGTATATGAAAAATAATAATAATTTTTTCAAGGGTATGGAAATAATTGAAGATCAAGAATTAGTTTCAAT
M K N N N N F F K G M E I I E D Q E L V S I
811 AACTGGAGGGAAAAAATGGGGCTGGCTAGCTTGGGTAGACCCAGCTTATGAATTTATCAAGGGCTTTGGTAAAGGTGCAAT
T G G K K W G W L A W V D P A Y E F I K G F G K G A I
892 TAAAGAAGGAAATAAAGATAAGTGGAAAAATATCTGACGAGTAACTTTTGTTAGATTAAATAAGGCAAGTAGTTTAGTTAA
K E G N K D K W K N I *
lagC
973 GCTATTTGCTTTTATTATGGTTGAAGAATTTACAAAAAGAAGGGATAACAATTTTTGTTTAATAATATAGTAGTTTTTATA
L F N N I V V F I

FIG.1 (2/6)

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1054  AATTTTTTAAGCTTTGTATTTATTCTTGTAGGTGTAGATATTAAATATAATGACAATCGGATAAAGATAGTACATGTTACT
      N  F  L  S  F  V  F  I  L  V  G  V  D  I  K  Y  N  D  N  R  I  K  I  V  H  V  T

1135  TTTTTTATTAGTTTCATTTTAGTAATGCTAACAAGTTTAATATCACATAATAGCATTGCATATAGCTTATCACAAATTTTG
      F  F  I  S  F  I  L  V  M  L  T  S  L  I  S  H  N  S  I  A  Y  S  L  S  Q  I  L

1216  GAAATTTTATGTATAATTTGTATTTTGCTTTTGTTTTATATTTTGAAAAAAACTAACTCACTAAGTAATAGAGCTAATGTG
      E  I  L  C  I  I  C  I  L  L  L  F  Y  I  L  K  K  T  N  S  L  S  N  R  A  N  V
                                                                    lacD
1297  GTATTTATTATTTTTATCGTTACTCAAGTTATTATAATCATAAATCAATTATTTATTAGGTGATTTTATGAAAAAAATAAT
      V  F  I  I  F  I  V  T  Q  V  I  I  I  I  N  Q  L  F  I  R  *     M  K  K  I  I

1378  ATATCAACAGGATGAAAAAGATTGTGGAGTAGCTTGTATAGCCATGATTTTAAAACATTATGGTACCGAAATTACTATTCA
      Y  Q  Q  D  E  K  D  C  G  V  A  C  I  A  M  I  L  K  H  Y  G  T  E  I  T  I  Q

1459  AAGGTTGCGTGAACTTTCTGGGACAGATTTAGATGGCACGTCTGCTTTTGGAATAAAAAAAACATTTGAAAAATTAGGATT
      R  L  R  E  L  S  G  T  D  L  D  G  T  S  A  F  G  I  K  K  T  F  E  K  L  G  F

1540  TGATGCACCAGCATTCAAAGCTGGTGATGAAACATGGCAAGAAAAAGATATACCCTTGCCTTTGATAGCTCACATAATAAG
      D  A  P  A  F  K  A  G  D  E  T  W  Q  E  K  D  I  P  L  P  L  I  A  H  I  I  S

1621  TGAACAAAAGTATCAACACTACGTAGTGGTTTATAAAGTTAAAGGTGATGAGATTTGGATTGCTGACCCAGCAAAGGGAAA
      E  Q  K  Y  Q  H  Y  V  V  V  Y  K  V  K  G  D  E  I  W  I  A  D  P  A  K  G  K

1702  GATTAGAAAAACTATTTCTGAATTTTCTAAAGAGTGGACAGGTGTCTTACTTTTTCCTAAACCAAAAGCAGAATACAAACC
      I  R  K  T  I  S  E  F  S  K  E  W  T  G  V  L  L  F  P  K  P  K  A  E  Y  K  P

1783  GTCTATTGAAAGAGTAGATAGTTTATCAACGTTCTTTCCTATACTAATAAAACAGAAGTCACTCTTCATCACGATTTTTGG
      S  I  E  R  V  D  S  L  S  T  F  F  P  I  L  I  K  Q  K  S  L  F  I  T  I  F  G

1864  AATCATAAGTTCTTACTATTTTCAAGGCTTATTGGATAATATTATTCCAAATCAGGCTCGGTCGACTTTAAATATTCTTTC
      I  I  S  S  Y  Y  F  Q  G  L  L  D  N  I  I  P  N  Q  A  R  S  T  L  N  I  L  S
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FIG. 1 (3/6)

```
1945  CATAGGACTTATTTTGTTTATCTTTTTCGTGTGCTTTTTGAATATAGTCGTAGCTATCTTCTACTTTTAATTGGGCAAAG
        I  G  L  I  F  V  Y  Y  L  F  F  R  R  V  L  F  E  E  Y  S  R  R  S  Y  Y  L  L  L  L  L  I  G  Q  R

2026  AATGAGTATGAGCATAATGCTTGGTTATTTTAAACACGTTTTGTCACTACCTCTGAGTTTCTTTGCAACCAGAAAGTCAGG
        M  S  M  S  I  M  L  G  Y  Y  F  K  H  V  L  S  L  P  L  S  F  F  A  T  R  K  S  G

2107  AGAAATTATTTCTCGCTTCTTGGATGCTAATAAAATTATTGATGCTCTTGCTAGTGCCACCTTATCTTTAATTCTGGATAT
        E  I  I  S  R  F  L  D  A  N  K  I  I  D  A  L  A  S  A  T  L  S  L  I  L  L  D  I

2188  TGGAATGGTAATTCTGGTTGGCACGACACTTGCGATTCAAAGTACTCAGCTTTTTTTGCTCACCCTTGCCTTTCTACCTTT
        G  M  V  I  L  V  G  T  T  L  A  I  Q  S  T  Q  L  F  L  L  T  L  A  F  L  P  F

2269  TTATATTTTGGTAGTATATGTGTTTATTAGGAGTTACGATAAAGCAAATACAGAAGAAATGAGTGCAGGAGCTGAAGTTAA
        Y  I  L  V  V  Y  V  F  I  R  S  Y  D  K  A  N  T  E  E  M  S  A  G  A  E  V  N

2350  TTCTAGTATTATTGAAAGTCTAAAAGGAATTGAAACTATTAAATCTTACAATGGAGAAAATCATGTCTATGATCGTGTAGA
        S  S  I  I  E  S  L  K  G  I  E  T  I  K  S  Y  N  G  E  N  H  V  Y  D  R  V  D

2431  TTCAGAATTTGTAACTTTAATGAAAAAGTCTTTTAAATCGGTCACACTTGATAATGTACAACAGAGTTTAAAAATGGTTAT
        S  E  F  V  T  L  M  K  K  S  F  K  S  V  T  L  D  N  V  Q  Q  S  L  K  M  V  I
```

FIG. 1 (4/6)

```
2512  TGAACTTATAAGTAGTGTATTGATACTATGGCTAGGGTCAAGTTATGTTATAGATGGAAAAATAAGTCTAGGACAATTAAT
        E  L  I  S  S  V  L  I  L  W  L  G  S  S  Y  V  I  D  G  K  I  S  L  G  Q  L  I

2593  TACCTATAATGCTTTACTTGTATTTTTTACTGAACCTTTACAAAATATTATTAATTTGCAAGTGAAAATGCAAAAAGCACG
        T  Y  N  A  L  L  V  F  F  T  E  P  L  Q  N  I  I  N  L  Q  V  K  M  Q  K  A  R

2674  TGTAGCAAATAAACGTTTGAACGAAATCATGTCAATATCTCCAGAACAAAGAAATACGAATATTAATATATCGAAAAATAT
        V  A  N  K  R  L  N  E  I  M  S  I  S  P  E  Q  R  N  T  N  I  N  I  S  K  N  I

2755  ATTTAATAAGGATATAAAATTAGATAAAGTAAGTTTTTCTTATAATATGAAGCTTCCCGTTTTAAGAGATGTTTCTTTAGA
        F  N  K  D  I  K  L  D  K  V  S  F  S  Y  N  M  K  L  P  V  L  R  D  V  S  L  E

2836  AATATATTCCAAAAGTAAGGTTGCTCTTGTTGGTGTGAGCGGTTCAGGCAAGTCTACACTAGCTAAACTATTAGTAAAATT
        I  Y  S  K  S  K  V  A  L  V  G  V  S  G  S  G  K  S  T  L  A  K  L  L  V  K  F

2917  CTATGATCCCTCTGAGGGAAATATCACTTATGGTGATATAAATTGTCAAGATATTGAAAATCATAAATTAAGAAATCATGT
        Y  D  P  S  E  G  N  I  T  Y  G  D  I  N  C  Q  D  I  E  N  H  K  L  R  N  H  V

2998  TACTTATGTTCCTCAGGAATCTTTCTTTTTTAATGGTACAATTATAGATAATTTAACTTTTGGTCTTAGTCATCAACCAGA
        T  Y  V  P  Q  E  S  F  F  F  N  G  T  I  I  D  N  L  T  F  G  L  S  H  Q  P  E

3079  GTTTGAAAAAATTTTTAGAGCATGTAAAGCTGCTTGTCTTGTTGATTTTATTAACCAACAACCTTTAAGATTTGATTCAGT
        F  E  K  I  F  R  A  C  K  A  A  C  L  V  D  F  I  N  Q  Q  P  L  R  F  D  S  V

3160  TCTTGAAGAAGGAGGAAATAATCTATCAGGAGGACAAAAGCAACGCTTAGCAATAGCTAGAGCTATTTTAAATGATTCTGA
        L  E  E  G  G  N  N  L  S  G  G  Q  K  Q  R  L  A  I  A  R  A  I  L  N  D  S  E

3241  AATAATTATTTTTGATGAAGCAACTAGTGGTCTTGATACCTTATTAGAAAAAGAGATTTTAGAATATTTAATTAAGTTACA
        I  I  I  F  D  E  A  T  S  G  L  D  T  L  L  E  K  E  I  L  E  Y  L  I  K  L  Q

3322  GGATAAAACTATCATTTTTATTGCCCACCATCTATCAATAGCTAAAGCCTGTGATGAAATCATTGTTCTAGATCAAGGAAT
        D  K  T  I  I  F  I  A  H  H  L  S  I  A  K  A  C  D  E  I  I  V  L  D  Q  G  I
```

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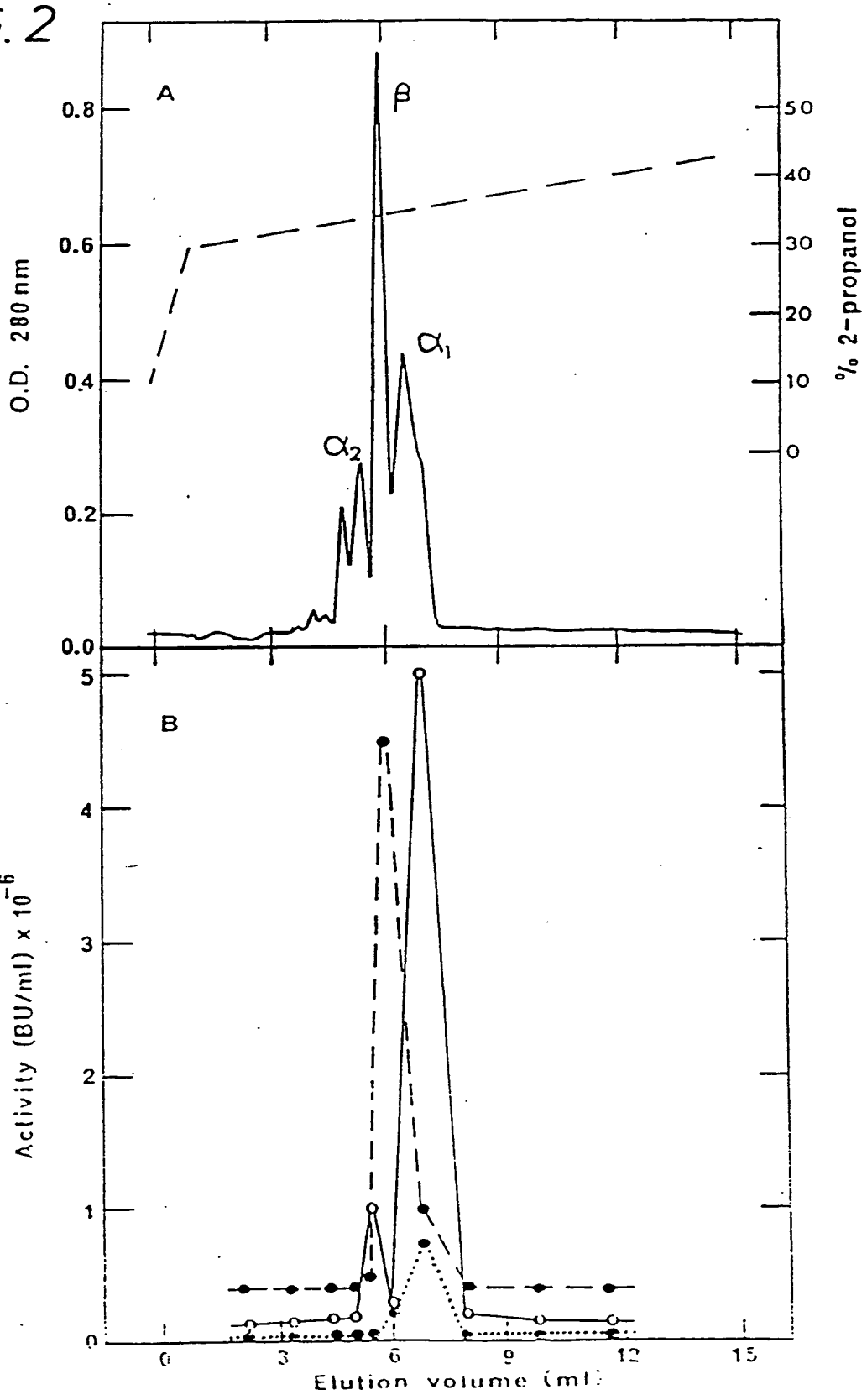
FIG. 1 (5/6)

```
3403  ATTGGTTGGGAGAGGAACACACGAAGAATTATCTGAAAAAGAGGGTGTATATAGGAGATTATTAAACGCATAACAACTTAC
        L  V  G  R  G  T  H  E  E  L  S  E  K  E  G  V  Y  R  R  L  L  N  A  *

                 lacE
3484  GGAGTAATACAAACATGAATCAAAATAATTGGCAAAATACTTCACAGTTCTATAGTCAAAGTCACAAACATTTCTATCGCT
                    M  N  Q  N  N  W  Q  N  T  S  Q  F  Y  S  Q  S  H  K  H  F  Y  R  W

3565  GGATTATTTATCCTACAGTAGTTTTATTTCTTTTTATTCTTCTTTTTTTATTCTTCGGGAAGAAAGAAATTATTATTAAAT
        I  I  Y  P  T  V  V  L  F  L  F  I  L  L  F  L  F  F  G  K  K  E  I  I  I  K  S

3646  CCCAAGCACAAATTACATCAGATTTAGTTTCTAAAATACAGATTCCAATTGAATCTAAAATAATAGAAAATAAACTTTCTG
        Q  A  Q  I  T  S  D  L  V  S  K  I  Q  I  P  I  E  S  K  I  I  E  N  K  L  S  E

3727  AAAATTTGTCTGTGAAAAAAGGGGAAGCATTAGTTAAGCTTGATATAGAGTCTCTTAAACAACAAAAAAGCAGTTTAGAAA
        N  L  S  V  K  K  G  E  A  L  V  K  L  D  I  E  S  L  K  Q  Q  K  S  S  L  E  K

3808  AGGAAATTCCTTTAGTTCAAAACCAAATTTTAGCTAATCAAACTTTCATTGATAGTCTGAAGCAAGGAAAAAGTTTGTTCA
        E  I  P  L  V  Q  N  Q  I  L  A  N  Q  T  F  I  D  S  L  K  Q  G  K  S  L  F  T

3889  CTTCAGATGATGATTTTGGTTATAGTAATCAATTGCAAAGCTTACTATCGGAGAATACTGCTTCTGATTATGCATTTAAAC
        S  D  D  D  F  G  Y  S  N  Q  L  Q  S  L  L  S  E  N  T  A  S  D  Y  A  F  K  Q

3970  AAAGCATAATTGATCATGAGAGTGAAAAAAGTACTTATCAAAATTCTAAAAATTCAATTCAGAAAAGCATCTCTAAAAAAC
        S  I  I  D  H  E  S  E  K  S  T  Y  Q  N  S  K  N  S  I  Q  K  S  I  S  K  K  Q

4051  AAGTAGAAAAAAATGATTGGGAAAAAATAAAAAGTGCTTGGAGTTCTCAGTCAAATCTTTCGGGGGCTTCAGAAGATACTA
        V  E  K  N  D  W  E  K  I  K  S  A  W  S  S  Q  S  N  L  S  G  A  S  E  D  T  T

4132  CTTCACAATATTTGAATTTTCAAGAACAACTAAAGGCTAGTTCAAAAGAAGAAAAAGAACAAGTAAAGGTAACTATTTCTT
        S  Q  Y  L  N  F  Q  E  Q  L  K  A  S  S  K  E  E  K  E  Q  V  K  V  T  I  S  S
```

FIG. 1 (6/6)

```
4213  CTAGTATTAATGATAAAATTTCACAAATTGACCAGGAATTGGAACAGTTAGAAATGGAGCAGTCAAAACTAACACCTCCAG
          S  I  N  D  K  I  S  Q  I  D  Q  E  L  E  Q  L  E  M  E  Q  S  K  L  T  P  P  A

4294  CTTCTTACGATAATGAGAAAAGTAGTCAGGAATATATAAAAAGAAACAGCTTGTTGAACAAACTATTGCAACGGCAAAGCAAA
          S  Y  D  N  E  K  S  S  Q  E  Y  K  K  K  Q  L  V  E  Q  T  I  A  T  A  K  Q  K

4375  AAAGAATAGAATTCAAAGAAGCGCAAGAAAAATATAATTTAGAACTTCAAGAAGTCAATAAGCAAATACAAGATGAAATAA
          R  I  E  F  K  E  A  Q  E  K  Y  N  L  E  L  Q  E  V  N  K  Q  I  Q  D  E  I  I

4456  TTACCTCACCTATTGATGGTTTTGTTCATATTAATTATAATGTCAAAGATCAGAAGATTATCCCTAAGGGAGAAGTTATTG
          T  S  P  I  D  G  F  V  H  I  N  Y  N  V  K  D  Q  K  I  I  P  K  G  E  V  I  A

4537  CCGAAATTTATCCAGAAATTAAACCAGGGAAAATTGAATTTACTTCCCAAATCGAAGCATCCGACTTAACACAAGTTAAAT
          E  I  Y  P  E  I  K  P  G  K  I  E  F  T  S  Q  I  E  A  S  D  L  T  Q  V  K  S

4618  CAGGGATGCCGGGTTCATTTTAAACTTGATTCAAAGGGAAATAGCCCTATCATAATGGAGGGAAAAATCAAAGAAATCTCTG
          G  M  R  V  H  F  K  L  D  S  K  G  N  S  P  I  I  M  E  G  K  I  K  E  I  S  A

4699  CGAATGCTGAAACTTCGGAGCGAGGAAGCTTTTATGTTGTAAAAGGGTTCTTAAAGCAGACAAATAAAACTCCTTTTAATA
          N  A  E  T  S  E  R  G  S  F  Y  V  V  K  G  F  L  K  Q  T  N  K  T  P  F  N  S

4780  GTCGATATGGCTTAAACGGTCGATTATCACTTATTGTTGGTAAAAAATCGTATTTTAATGTTTTAAAGAAATGATTATAA
          R  Y  G  L  N  G  R  L  S  L  I  V  G  K  K  K  S  Y  F  N  V  L  K  E  M  I  I  K

4861  AAACTAAAAATATCTAGTTTTTAATTAGCAGTTTATCACATGTAACAATTTACAAAA
          N  *
```

FIG. 2


A
β
α1
α2
O.D. 280 nm
% 2-propanol
0.0
0.2
0.4
0.6
0.8
0
10
20
30
40
50
B
Activity (BU/ml) x 10⁻⁶
0
1
2
3
4
5
0
3
6
9
12
15
Elution volume (ml)

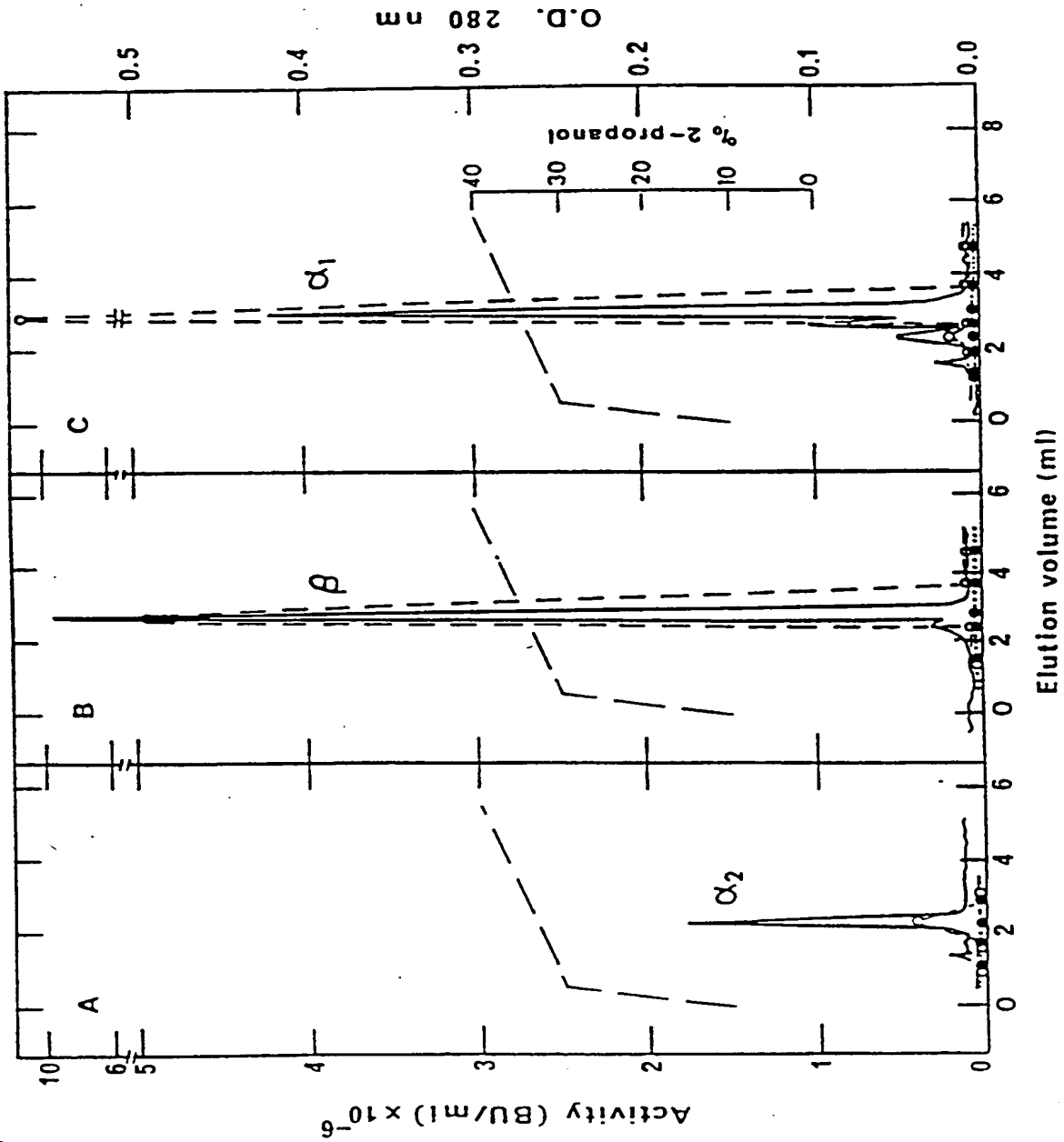

O.D. 280 nm
0.5
0.4
0.3
0.2
0.1
0.0
% 2-propanol
40
30
20
10
0
α_1
β
α_2
A
B
C
Activity (BU/ml) x 10^{-6}
Elution volume (ml)

FIG. 3

FIG. 4

α_1 and α_2:

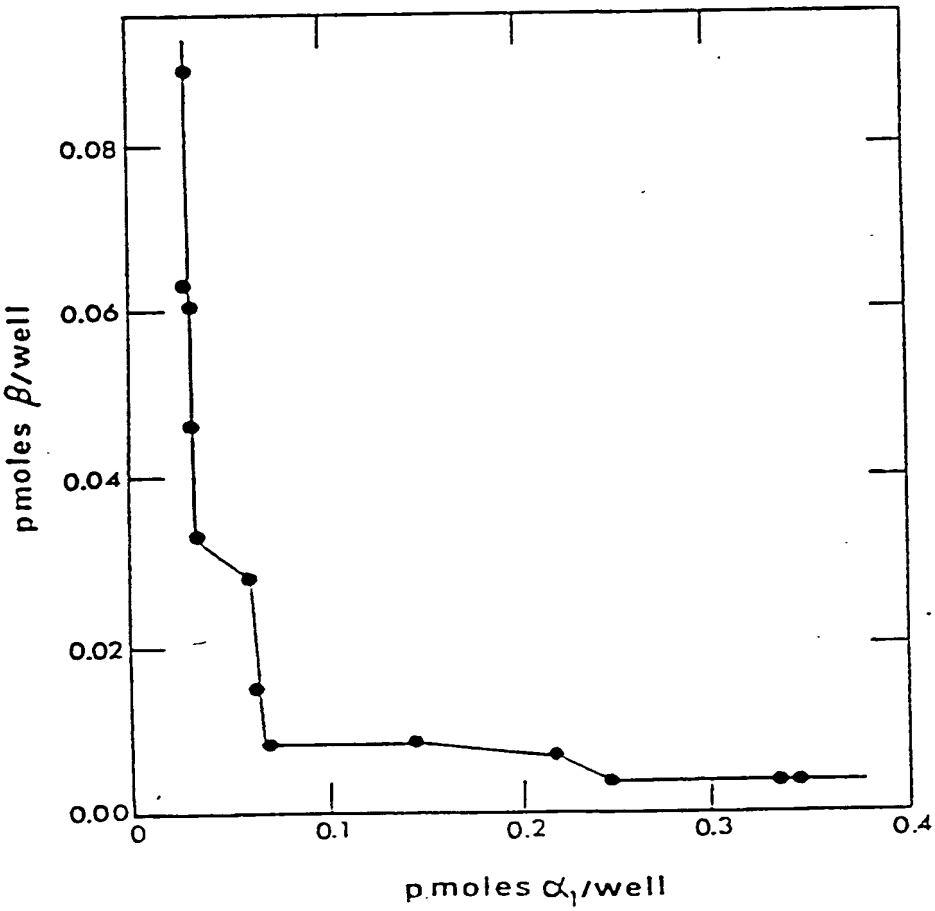
N	Gly	Thr	Trp	Asp	Asp	Ile	Gly	Gln	Gly
	Ile	Gly	Arg	Val	Ala	Tyr	Trp	Val	Gly
	Lys	Ala	Met	Gly	Asn	Met	Ser	Asp	Val
	Asn	Gln	Ala	Ser	Arg	Ile	Asn	Arg	Lys
	Lys	Lys	His	C					

β:

N	Lys	Lys	Trp	Gly	Trp	Leu	Ala	Trp
	Val	Asp	Pro	Ala	Tyr	Glu	Phe	Ile
	Lys	Gly	Phe	Gly	Lys	Gly	Ala	Ile
	Lys	Glu	Gly	Asn	Lys	Asp	Lys	Trp
	Lys	Asn	Ile	C				

SUBSTITUTE SHEET

FIG. 5


0.08
0.06
0.04
0.02
0.00
pmoles β/well
0
0.1
0.2
0.3
0.4
p.moles α_1/well

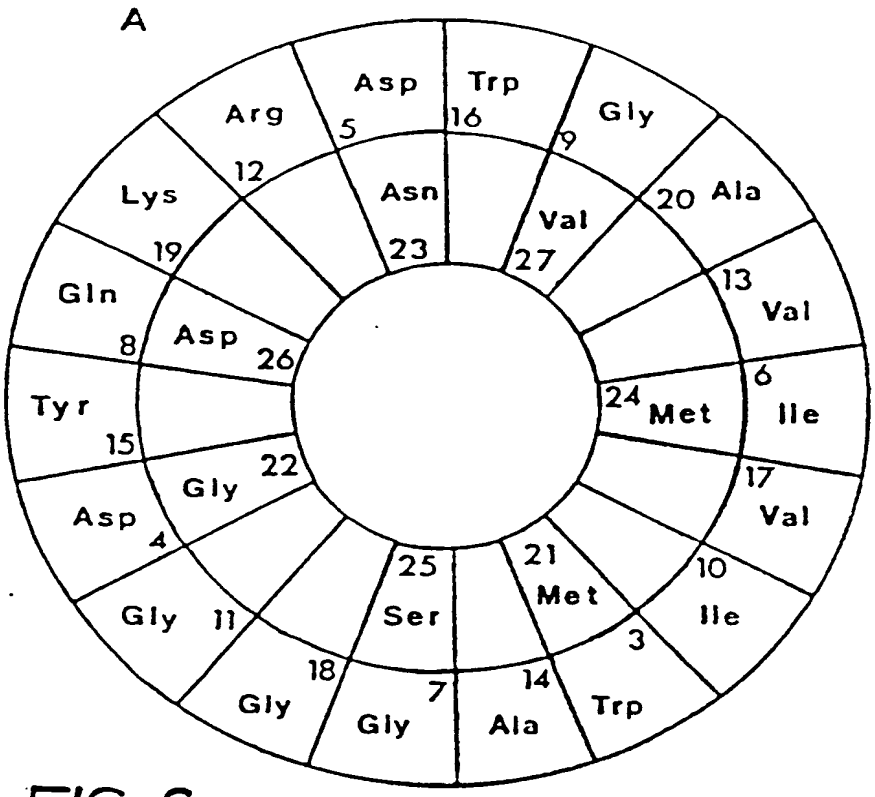
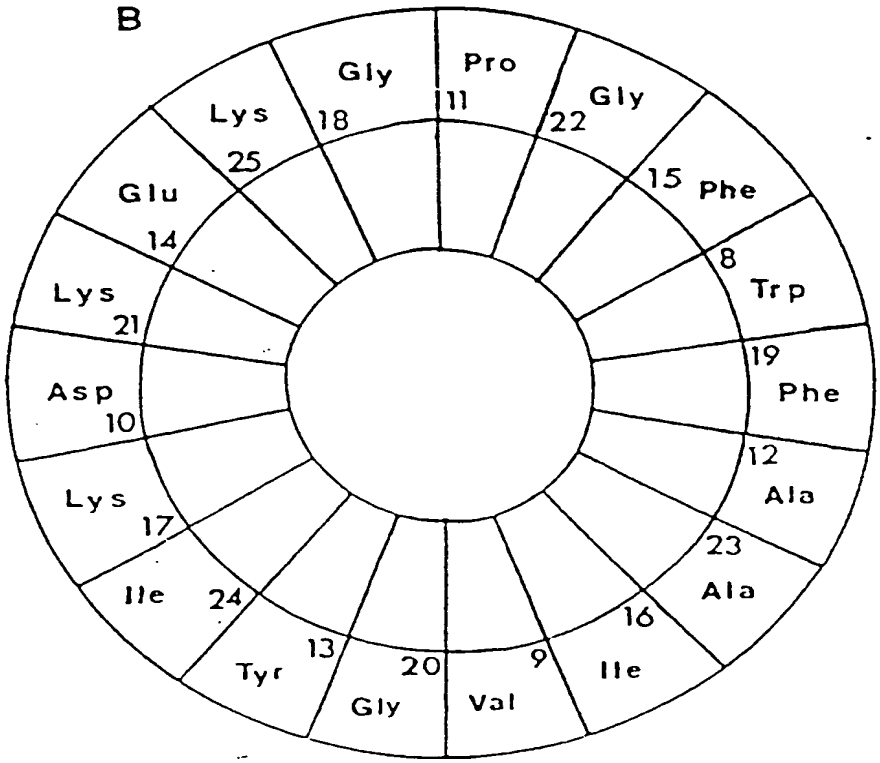

A
Asp 5
Trp 16
Gly 9
Arg 12
Asn 23
Val 27
Ala 20
Lys 19
Val 13
Gln 8
Asp 26
Met 24
Ile 6
Tyr 15
Val 17
Gly 22
Asp 4
Met 21
Ile 10
Ser 25
Gly 11
Trp 3
Gly 18
Gly 7
Ala 14

FIG. 6


B
Gly 18
Pro 11
Gly 22
Lys 25
Phe 15
Glu 14
Trp 8
Lys 21
Phe 19
Asp 10
Ala 12
Lys 17
Ala 23
Ile 24
Ile 16
Tyr 13
Val 9
Gly 20

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 93/01799

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/31 C07K13/00 C12P21/02 A23C19/032 A23C9/123
C12N15/74 C12N1/20 A01K67/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07K C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	J. BACTERIOL. vol. 174, no. 17 , September 1992 pages 5686 - 5692 NISSEN-MEYER, J. ET AL. 'A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides' see the whole document --- -/--	1-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
22 December 1993	24 -01- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Espen, J

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 93/01799

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	APPLIED AND ENVIRONMENTAL MICROBIOLOGY vol. 58, no. 2 , February 1992 pages 572 - 577 BELKUM VAN, M. J. ET AL. 'Cloning, sequencing, and expression in Escherichia coli of lcnb, a third bacteriocin determinant from lactococcal bacteriocin plasmid p9B4-6' see the whole document see figures 1,2	1-7, 13-19
Y	APPLIED AND ENVIRONMENTAL MICROBIOLOGY vol. 57, no. 2 , February 1991 pages 492 - 498 BELKUM VAN, M. J. ET AL. 'Organization and nucleotide sequences of two lactococcal bacteriocin operons' see the whole document see figures 3,5-6	1-7, 13-19
Y	APPLIED AND ENVIRONMENTAL MICROBIOLOGY vol. 55, no. 5 , May 1989 pages 1187 - 1191 BELKUM VAN, M. J. ET AL. 'Cloning of two bacteriocin genes from a lactococcal bacteriocin plasmid' see page 1188 - page 1189	1-7, 13-19
A	WO,A,91 19802 (NORWEGIAN DAIRIES ASSOCIATION) 26 December 1991 see the whole document	1-19

1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 93/01799

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9119802	26-12-91	AU-A- 8081691	07-01-92
		EP-A- 0535039	07-04-93